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Photoinduced electron transfer in ruthenium-modified cytochrome *c*

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ABSTRACT: **Distant heme-Ru electronic couplings have been extracted from intramolecular electron-transfer rates in Ru(histidine-X) (X=33,39,62) derivatives of cytochrome *c*. The rates (and the couplings) correlate with the lengths of σ-tunneling pathways comprised of covalent bonds, hydrogen bonds, and through-space jumps from the histidines to the heme group.**

The electron-transfer (ET) reactions that occur within and between proteins typically involve prosthetic groups separated by distances that are often greater than 10 Å. An understanding of how the intervening medium, driving force, and nuclear reorganization energetics and dynamics modulate protein ET reactions has been a central goal of our research program. In recent years, we have been examining the rates of electron transfer between surface-bound ruthenium complexes and metalloprotein active sites.[1] This work has provided a considerable capacity for predicting protein ET rates.

We have been guided by semiclassical ET theory, which describes the rate constant for nonadiabatic reaction between a donor and acceptor held at fixed distance and orientation:[2]

$$k_{ET} = (\pi/\hbar^2\lambda k_B T)^{1/2}(H_{AB})^2 \exp\{-(\Delta G^\circ + \lambda)^2/4\lambda k_B T\} \qquad (1)$$

The tunneling matrix element H_{AB} is a measure of the electronic coupling between the reactants and the products at the transition state. The magnitude of H_{AB} depends upon donor-acceptor separation, orientation, and the nature of the intervening medium. The exponential term in Eq. 1 reflects the interplay between reaction driving force ($-\Delta G^\circ$) and nuclear reorganization energy (λ). Various approaches have been used to test the validity of Eq. 1, and to extract the ET parameters H_{AB} and λ. Driving-force studies have proven to be a reliable approach, and such studies have been emphasized in our own work.

In the nonadiabatic limit, the probability is quite low that reactants will cross over to products at the transition-state configuration.[2] This probability depends upon the electronic hopping frequency (determined by H_{AB}) and upon the frequency of motion along the reaction coordinate.[3] When solvent reorientation dominates λ, the nuclear reorientation timescale is believed to be given by the solvent longitudinal dielectric relaxation time, τ_L. The nonadiabatic limit for ET results when $H_{AB}^2 \ll \{\lambda_s\hbar/4\pi\tau_L\}^{1/2}$.[3] Water reorients very rapidly ($\tau_L \approx 0.5$ ps[4]) and the solvent-controlled adiabatic limit results when $H_{AB} \gg 80$ cm^{-1}. Conversely, when $H_{AB} \ll 80$ cm^{-1}, Eq. 1 should adequately describe the ET kinetics. Reorientation of the peptide matrix introduces complications in protein ET. Timescales for this nuclear motion are much slower than the τ_L for water.[5] In situations where slow peptide motions dominate λ, much smaller values of H_{AB} are necessary to achieve the "solvent-controlled" adiabatic limit.

In simple models, the electronic-coupling strength is predicted to decay exponentially with increasing donor-acceptor separation (Eq. 2):[2,6]

$$H_{AB} = H_{AB}^\circ \exp\{-\tfrac{1}{2}\beta(d-d^\circ)\} \qquad (2)$$

In Eq. 2, H_{AB}° is the electronic coupling at close contact (d°), and β is the rate of decay of coupling with distance (d). Studies of the distance dependence of ET rates in donor-acceptor complexes, and of randomly oriented donors and acceptors in rigid matrices, have suggested $0.8 \le \beta \le 1.2$ Å^{-1}.[7-12] Donor-acceptor electronic coupling in small complexes can be interpreted equally well in terms of simple exponential decay with distance (Eq. 2) or with the number of chemical bonds in the bridge between redox sites.[8] This situation arises from the fact that the direct distance between redox sites tends to be

proportional to the number of intervening chemical bonds. The medium separating two redox sites in a protein, however, is a heterogenous array of bonded and nonbonded interactions. The covalently bonded path between donor and acceptor can be a tortuous route involving many more bonds than would be found in a typical synthetic D-A complex with comparable separation. Beratan and Onuchic have developed a formalism that describes the medium between redox sites in a protein in terms of "unit blocks" connected together to form a physical pathway for ET.[13] A unit block may be a covalent bond, a hydrogen bond, or a through-space jump, each with a corresponding decay factor. At intermediate D-A distances, a single pathway tends to dominate the coupling and H_{AB} can be written as the product of the decay factors for each block in the pathway. By scaling H-bonds and through-space jumps to the number of covalent bonds that would give a comparable decay in coupling strength, pathways can be described in terms of a number of *effective bonds* (n_{eff}) between the redox sites.

Work in our laboratory on intramolecular protein ET reactions began with experiments in which horse heart cytochrome *c* was modified by coordination of pentaammineruthenium to His33 (Figure 1).[14,15] The rate of intramolecular ET from $Ru_5(His33)^{2+}$ to the ferriheme (T = 298 K), measured using photochemical techniques, is 30(5) s^{-1} (Table I).[14] The reaction exhibits a rather small activation enthalpy (2 kcal mol^{-1}), and a large negative activation entropy (-43 eu). Measurements of the temperature dependences of the $Ru_5(His)^{3+/2+}$ and $Fe^{3+/2+}$ potentials in $Ru_5(His33)$-Fe-cyt *c* have provided estimates of $\Delta G°$(-4.3(2) kcal mol^{-1}, 298 K), $\Delta H°$ (-11.9(10) kcal mol^{-1}), and $\Delta S°$ (-26(3) eu) for the $Ru^{II} \rightarrow Fe^{III}$ intramolecular ET reaction. Given these thermodynamic quantities, and the temperature dependence (2-40 °C) of the ET rate in $Ru_5(His33)$-Fe-cyt *c*, it is possible to extract values of λ and H_{AB} from Eq. 1. Nonlinear least-squares fits to the data suggest λ=1.2 eV and H_{AB}=0.03 cm^{-1}.[16] This value of the reorganization energy is quite close to that predicted by the Marcus cross relation[2] ($\lambda_{12} = \frac{1}{2}(\lambda_{11}+\lambda_{22})$) using the reorganization energies for the Fe(III/II)-cyt *c* (λ_{11} = 1.04 eV) and $Ru_5(py)^{3+/2+}$ (λ_{22} = 1.20 eV) self-exchange reactions.[2,17]

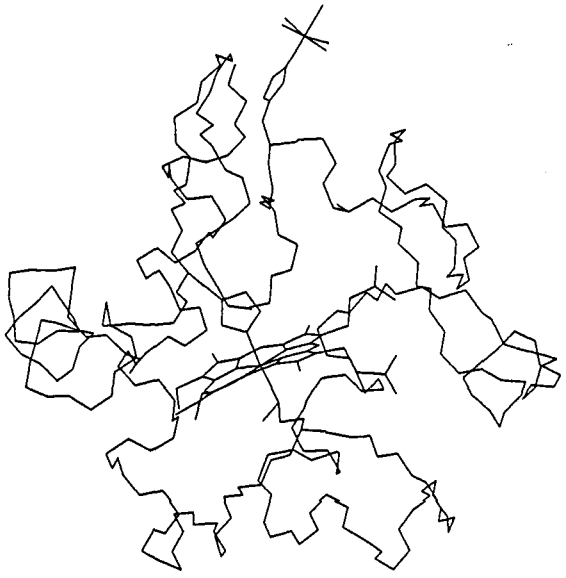

Figure 1. Peptide-backbone structure of $Ru_5(His33)$Fe-cyt *c*.

A value of β = 2.0 $Å^{-1}$ can be extracted from Eq. 2 for the $Ru_5(His33)$-Fe-cyt *c* system by taking **d** = 11.1 Å,[18] **d°** = 3.0 Å,[2] and assuming that $H°_{AB}$ = 200 cm^{-1}.[16] The large value of β suggested by the $Ru_5(His33)$-Fe-cyt *c* temperature-dependence data indicates a faster decay of electronic coupling with donor-acceptor separation than found in small-molecule systems. Alternatively, the large apparent β could suggest that, owing to the inhomogeneity of the intervening medium, the effective distance for ET is greater than the 11.1-Å direct separation. These early data, then, provided some evidence that donor-acceptor electronic coupling in protein systems might not be described by the simple expression in Eq. 2.

A clear understanding of the electronic-coupling strengths in metalloprotein ET reactions depends upon reliable values of λ and H_{AB}. It is clear from Eq. 1 that, in addition to studies of temperature dependences, ET parameters can also be extracted from studies of the driving-force dependence of ET rates. In the low-driving-force regime ($-\Delta G°/\lambda \ll 1$), the variation of rate with free energy does not strongly depend upon λ (i.e., $\partial(\ln k_{ET})/\partial(\Delta G°) \approx 1/2k_BT$), and it is difficult to obtain a good value for this parameter. Better values of λ and H_{AB} can be obtained from high-driving-force measurements (i.e., $\lambda \approx -\Delta G°$). In this region, the driving-force curve flattens out and ET rates approach their maximum values.

It is difficult to prepare a Ru-ammine complex of Fe-cyt *c* in which the driving force for intramolecular ET is much greater than 0.2 eV. Substitution of the native Fe center in cytochrome *c* with Zn,[19] however, has provided an avenue to high-driving-force intramolecular ET. The lowest triplet excited state of the Zn-porphyrin in Zn-cyt *c* has a 15-ms lifetime and is a potent reductant ($E° = -0.62$ V *vs.* NHE).[20] The rates of direct photoinduced ET and thermal recombination have been measured for three Ru_5L(His33)-Zn-cyt *c* proteins (L = NH_3, pyridine, isonicotinamide), spanning a 0.39-eV range in $\Delta G°$ (-0.66 to -1.05 eV, Table I).[20-22] Fits of these data to Eq. 1 yield $\lambda = 1.10$ eV and $H_{AB} = 0.12$ cm^{-1} for the photoinduced reactions, and $\lambda = 1.19$ eV and $H_{AB} = 0.09$ cm^{-1} for the recombinations. The ET parameters are not extremely sensitive to the nature of the reaction (photoinduced or recombination), and these reactions adequately can be described by a single pair of parameters: $\lambda = 1.15(10)$ eV and $H_{AB} = 0.1(2)$ cm^{-1} (Figure 2). The value of H_{AB} in Ru(His33)-Zn-cyt *c* is about three times that estimated for Ru(His33)-Fe-cyt *c*. The difference may be the result of variations in coupling to Ru between the porphyrin-localized states in Zn-cyt *c* and the metal-localized states in Fe-cyt *c*.

Table I. Rate constants and activation parameters for intramolecular ET reactions of Ru(His)-modified cytochrome *c*.

Electron Transfer	$-\Delta G°$ *(eV)*	k_{ET} *(s^{-1})*	$\Delta H^{\ddagger}$ *(kcal mol^{-1})*	$\Delta S^{\ddagger}$ *(eu)*
His33 Derivatives (d = 11.1 Å) [a]				
$Ru_5(His)^{2+} \rightarrow Fe^{III}$ [b]	0.18(2)	$3.0(5) \times 10^{1}$	2.0(5)	-43(5)
$Ru_4(isn)(His)^{2+} \rightarrow ZnP^{+}$ [c]	0.66(5)	$2.0(2) \times 10^{5}$	<0.5	-35(5)
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$ [d]	0.70(5)	$7.7(8) \times 10^{5}$	1.7(4)	-27(5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$ [c]	0.74(5)	$3.5(4) \times 10^{5}$	<0.5	-34(5)
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$ [c]	0.97(5)	$3.3(3) \times 10^{6}$	2.2(4)	-22(5)
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$ [d]	1.01(5)	$1.6(4) \times 10^{6}$	--	--
$ZnP^{*} \rightarrow Ru_4(isn)(His)^{3+}$ [c]	1.05(5)	$2.9(3) \times 10^{6}$	<0.5	-30(5)
His39 Derivatives (d = 12.3 Å) [a,c]				
$Ru_4(isn)(His)^{2+} \rightarrow ZnP^{+}$	0.66(5)	$6.5(7) \times 10^{5}$	-1.7(4)	-39(5)
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$	0.70(5)	$1.5(2) \times 10^{6}$	1.3(3)	-27(5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$	0.74(5)	$1.5(2) \times 10^{6}$	-1.8(4)	-37(5)
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$	0.97(5)	$8.9(9) \times 10^{6}$	0.2(2)	-27(5)
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$	1.01(5)	$5.7(6) \times 10^{6}$	-0.2(2)	-29(5)
$ZnP^{*} \rightarrow Ru_4(isn)(His)^{3+}$	1.05(5)	$1.0(1) \times 10^{7}$	0.2(2)	-27(5)
His62 Derivatives (d = 14.8 Å) [a]				
$Ru_5(His)^{2+} \rightarrow Fe^{III}$ [f]	0.20(2)	1.7(1)	--	--
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$ [g]	0.70(5)	$6.5(7) \times 10^{3}$	1.4(3)	-37(5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$ [g]	0.74(5)	$8.1(8) \times 10^{3}$	--	--
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$ [g]	0.97(5)	$3.6(4) \times 10^{4}$	--	--
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$ [g]	1.01(5)	$2.0(2) \times 10^{4}$	0.7(7)	-37(5)

[a] Reference 18. [b] Reference 14. [c] Reference 21. [d] Reference 20. [e] Reference 22. [f] Reference 26. [g] Reference 27.

Table II. Maximum rates, D-A distances, coupling strengths, and effective bonds in pathways for Ru_4L(His)-modified cytochrome *c*.

	k_{MAX} (s^{-1})	d [a] (Å)	H_{AB} (cm^{-1})	n_{eff} [b] (bonds)
His39 [c]	1.4×10^{7}	12.3	0.24	14.0
His33 [d]	2.9×10^{6}	11.1	0.11	13.9
His62 [e]	2.0×10^{4}	14.8	0.01	20.6

[a] Reference 18. [b] Reference 16. [c] Reference 22. [d] Reference 21. [e] Reference 27.

The similarity in reorganization energies for the Ru-Fe-cyt *c* and Ru-Zn-cyt *c* intramolecular ET reactions is to be expected. The total reorganization energy is a sum of inner-sphere (λ_i) and outer-sphere (λ_o) elements. Inner-sphere contributions arise from nuclear rearrangements in the Ru-ammine and metalloporphyrin complexes accompanying electron transfer. These rearrangements are rather small and have been estimated to contribute no more than 0.2 eV to λ for both Ru-Fe-cyt *c* and Ru-Zn-cyt *c*.[21] There are two sources of outer-sphere rearrangements: the solvent and the peptide matrix. Calculations based on a single-sphere dielectric continuum model[23] indicate a 0.6-eV contribution to λ_o from the solvent.[21] From the structures of ferri- and ferrocytochromes *c*, the peptide contribution to λ_o has been calculated to be about 0.2 eV.[24] The sum of these individual components (1.0 eV) is in good agreement with the experimentally derived reorganization energy for the Ru-M-cyt *c* (M = Fe, Zn) systems.

Ru-ammine complexes also have been bound to His39 of Zn-substituted cytochrome *c* from *Candida krusei*.[22,25] Intramolecular ET rates (Table I) are approximately three times faster than those of corresponding reactions in His33 derivatives of horse heart cytochrome *c*. The variation of rates with driving force in these derivatives suggests a 1.2(1)-eV reorganization energy, indistinguishable from that found in the His33-modified proteins. The faster ET rates have been attributed to stronger donor-acceptor electronic coupling in the His39-modified protein.[22]

The direct D-A distances in Ru(His33)-Zn-cyt *c* and Ru(His39)-Zn-cyt *c* are at variance (11.1 and 12.3 Å, respectively) with the twofold larger H_{AB} for the His39 system. The pathway model is somewhat more consistent with the data: both the His33 and His39 pathways consist of 11 covalent bonds and 1 H-bond (Figure 3). The n_{eff} values for His33 and His39 are 13.9 and 14.0 bonds, respectively.[16]

Site-directed mutagenesis creates many new opportunities for studying electron transfer in Ru-modified

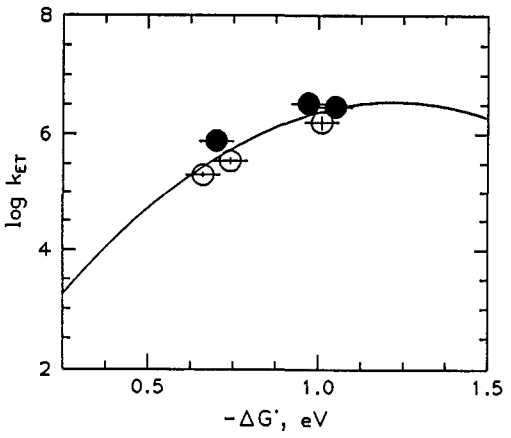

Figure 2. Free-energy dependence of intramolecular ET rates in Rua_4L(His33)-Zn-cyt *c*. Filled symbols: photoinduced reactions. Open symbols: recombination reactions. Solid line: best fit to Eq. 1.

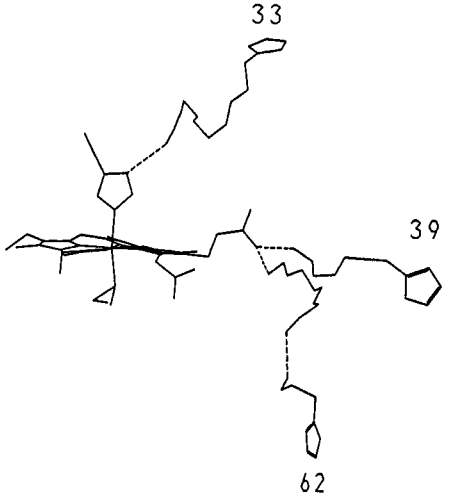

Figure 3. Predicted electronic coupling pathways in Ru(His33)-, Ru(His39)-, and Ru(His62)-modified cytochrome *c*. Solid lines: covalent bonds. Dashed lines: H-bonds.

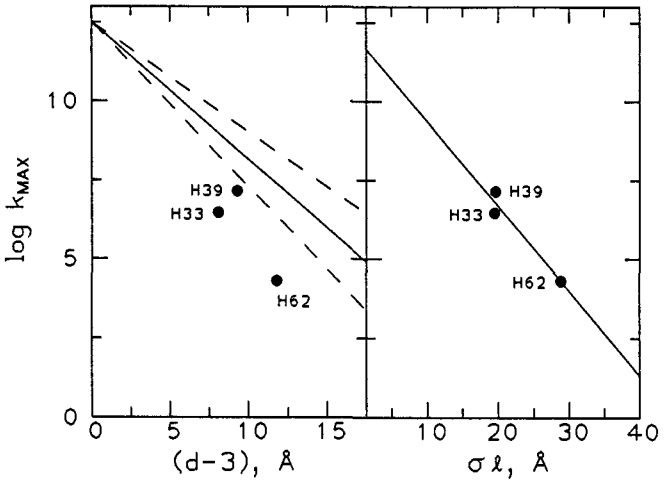

Figure 4. Plots of maximum ET rates in Ru(HisX)-cyt *c versus* **d** (left) and $\sigma\ell$ (right).

proteins. A yeast (*Saccharomyces cerevisiae*) cytochrome *c* variant has been characterized with a surface histidine at position 62.[26] The Ru_5(His62) derivative of this mutant protein was prepared, and the rate of electron transfer from Ru^{II} to Fe^{III} was found to be 1.7 s^{-1} (Table I).[26] Ru_5(His62) and Ru_4(py)(His62) derivatives of Zn-substituted *S.c.* cytochrome *c* have also been examined. The rates of the photoinduced and thermal recombination reactions are more than two orders of magnitude slower than the rates of analogous reactions in His33 derivatives of horse heart cytochrome *c*.[27] The driving-force data are more limited than for the other His derivatives of cytochrome *c*, but again suggest that $\lambda \approx 1.2$ eV. The slower rates for the His62 derivatives are attributed to weaker electronic coupling. The direct D-A separation is 14.8 Å, while the effective number of bonds in the pathway is 20.6 (Figure 3).[16] By both measures, it is reasonable to expect the His62 ET reactions to be substantially slower than those found in His33 or His39 derivatives.

Based on the few systems in which a reliable number has been extracted, $\lambda = 1.2$ eV appears to be a reasonable value for Ru-ammine-modified proteins. Perhaps due to lack of data and limited precision in the derived parameters, λ has not been found to be particularly sensitive to D-A separation or site of modification. In fact, the simple Marcus cross relation provides a reasonably good estimate of the reorganization energies in these reactions. Since outer-sphere reorganization seems to dominate, changes in the Ru-coordination sphere (ammine → bipyridine) appear to have the greatest impact on λ.

Unlike the reorganization energy, the electronic-coupling strengths in the Ru-modified proteins show a great deal of variability. Eq. 2 expresses a simple distance dependence for H_{AB} that adequately describes ET in model D-A complexes with values of β between 0.8 and 1.2 $Å^{-1}$. This distance dependence, assuming a maximum ET rate of 10^{13} s^{-1} at close contact ($\mathbf{d} = 3$ Å), is represented by the solid ($\beta = 1.0$ $Å^{-1}$) and dashed ($\beta = 0.8, 1.2$ $Å^{-1}$) lines in the left panel of Figure 4. In this panel the maximum ET rates (i.e., the rate at $-\Delta G° = \lambda$) for Ru-modified cytochromes *c* (Table II) are plotted as a function of D-A separation. It is clear that all of the maximum rates lie below the values predicted by Eq. 2, and that there is no simple correlation. The obvious conclusion is that, for a given D-A separation, the electronic coupling in the protein is substantially weaker than predicted by a simple exponential decay with distance.

The Beratan-Onuchic pathway model predicts the failure of exponential-decay correlations based on edge-edge distances. According to this model, maximum ET rates correlate with the effective number of bonds in the pathway. (Multiplying n_{eff} by a canonical value of 1.4 Å/bond gives a tunneling length ($\sigma\ell$) that replaces **d** in rate-distance correlations.) Maximum ET rates in the three Ru-modified cytochromes *c* are plotted against $\sigma\ell$ in the right panel of Figure 4. A linear least-squares fit to these three points gives the solid line with a slope of 0.6 $Å^{-1}$. Though the data are limited, it is important to note that the intercept at 1 bond (i.e., 1.4 Å) corresponds to a maximum ET rate of 4.6×10^{11} s^{-1}, which is in reasonable agreement with results from complexes with short D-A separations.[28-30]

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